Stereoselective fatty acylation is essential for the release of lipidated WNT proteins from the acyltransferase Porcupine (PORCN)

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The maintenance of adult animal tissues depends upon highly conserved intercellular signaling molecules that include the secreted WNT proteins. Although it is generally accepted that lipidation of WNTs by the acyltransferase Porcupine (PORCN) and their subsequent recognition by the Wntless (WLS) protein is essential for their cellular secretion, the molecular understanding of this process remains limited. Using structurally diverse fatty acyl donor analogs and mouse embryonic fibroblasts expressing PORCN protein from different metazoan phyla, we demonstrate here that PORCN active-site features, which are conserved across the animal kingdom, enforce *cis*-Δ9 fatty acylation of WNTs. Aberrant acylation of a WNT with an exogenously supplied *trans*-Δ9 fatty acid induced the accumulation of WNT-PORCN complexes, suggesting that the fatty acyl species is critical for the extrication of lipidated WNTs from PORCN. Our findings reveal a previously unrecognized fatty acyl–selective checkpoint in the manufacturing of a lipoprotein that forms a basis for WNT signaling sensitivity to *trans* fats and to PORCN inhibitors in clinical development.

Multicellular organisms that generate specialized tissues and organs must balance the needs of its constituent cells with those of the collective. Mechanisms that support the integration of metabolic processes and cell–cell communication provide a direct means for synchronizing efforts that meet these demands. For example, immobilization of lipids onto proteins that function in cell signaling can dictate protein distribution in the intra- and extracellular milieu or directly gate protein (1).

The WNT proteins control cell fate decision-making in diverse tissues during development and homeostatic renewal (2, 3). Animals typically express multiple WNTs that require lipidation for their secretory pathway transit and ultimate release into the extracellular milieu (4). Among the three characterized lipidated extracellular proteins, which also include the Hedgehog and Ghrelin proteins, WNT proteins are unique in their subjection to this production checkpoint. This phenomenon involves a polytopic protein known as WLS,[4] which fails to ferry WNT proteins to the extracellular space in the absence of PORCN activity (5, 6). How WLS distinguishes between an acylated and naked WNT protein remains unknown.

PORCN is the founding member of the membrane-bound *O*-acyltransferase (MBOAT) gene family, which consists of 16 polytopic acyltransferases with established lipid and protein substrates (7–9). PORCN is the only enzyme that has been shown to covalently attach a monounsaturated fatty acid (MUFA) onto proteins. Given that the lipid adduct identity has only been determined in a single mammalian WNT protein, it remains unclear whether it varies depending on species-specific metabolic considerations or whether evolutionary constraints have eliminated lipid diversity. Indeed, the mechanistic basis for WNT production dependence on MUFA acylation remains elusive.

The recent advance of PORCN inhibitors in clinical testing has galvanized efforts to gain structural insights into the basis of chemical specificity and, more broadly, a mechanistic understanding of why loss of lipidation cripples WNT production (10–12). Here, using a cross-species PORCN interrogation platform and chemical probes that target the PORCN active site, we reveal that PORCN harbors features conserved across diverse animals that enforce MUFA modification of WNTs. We further show that this enforcement is coupled to the ability of lipidated WNT proteins to extricate from PORCN and transfer to WLS. Our studies reveal a previously unrecognized checkpoint for fatty acyl selectivity in WNT manufacturing that is the target of *trans* fats and synthetic inhibitors of PORCN.

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[4] The abbreviations used are: WLS, Wntless; PORCN, Porcupine; MBOAT, membrane-bound *O*-acyltransferase; MUFA, monounsaturated fatty acid; cDNA, complementary DNA; ConA, concanavalin A; SCD, stearoyl-CoA desaturase; FA, fatty acid; GL, *Gaussia* luciferase; HHAT, hedgehog acyltransferase; SHH, sonic hedgehog.

Results

Conservation of MUFA labeling in WNT proteins

Despite the recognition that WNTs are found in all metazoans, it is unclear whether this conservation extends to the nature of the covalently attached lipid moiety. To address this question, we assembled a collection of PORCN cDNAs from diverse metazoan phyla that, in some cases, reach more than 70% sequence divergence, providing a means to broadly evaluate PORCN fatty acyl donor preferences within the animal kingdom (Fig. 1A and Fig. S1). Next, we built on an assay described previously using *PORCN*-null mouse embryonic fibroblasts expressing a murine WNT3A protein to first evaluate the ability of various PORCN molecules to induce WNT signaling (13) (Fig. 1B). This approach affords a controlled experimental platform focused on a single WNT substrate while removing species-or cell line–specific lipid metabolic considerations from the observations. We then established that the PORCN proteins evaluated were indeed capable of promoting murine WNT3A activity, as measured using either the synthetic WNT pathway reporter described above (Fig. 1, B and C) or phosphorylation of Dishevelled 2 (DVL2), a Frizzled (FZD) receptor–regulated signaling molecule (Fig. 1, B, D, and E). A PORCN inhibitor, WNT974, suppressed pathway activity mediated by PORCN from multiple sources, barring the activity mediated by *Drosophila* and *Schistosoma* PORCNs. These observations suggest a minimal WNT sequence requirement for maintaining an enzyme–substrate relationship. Indeed, PORCN is able to lipidate the disulfide-bonded β-hairpin that harbors the conserved acylated serine in WNT proteins when it is expressed in the context of a structurally distinct protein scaffold (14).

Having established a platform for measuring the activity of PORCN protein from diverse animals using the same cellular backdrop and a shared WNT substrate, we then evaluated the preference of various PORCN proteins for alkynylated palmitate (C16:0) or palmitoleate (C16:1n-7) (15). Observed for every PORCN protein tested was abundant labeling of the WNT protein with the palmitoleate but not the palmitate analog (Fig. 1F). These observations, which include PORCN from 4 of the 9 animal phyla and representative animals that diverged ~1.2 billion years ago (between Nematoda and Chordata) (16), suggest that PORCN universally enforces a monounsaturated fatty acyl donor preference in metazoans. Fatty acid desaturation is crucial for the labeling of multiple WNT proteins, as A939572, an inhibitor of stearoyl-CoA desaturase (SCD), which converts palmitate to palmitoleate, blocked WNT lipidation (Fig. 1G and Fig. S2). IWP2, a PORCN inhibitor structurally distinct from WNT974 (17, 18), also inhibited WNT palmitoleation.

### A universal carbon-counting mechanism found in PORCN enforces WNT modifications with a MUFA

Although animals typically harbor multiple WNT genes, only a single WNT protein produced from an isolated cell line has been subjected to mass spectrometric analysis with the intention of identifying the adducted lipid (19). At the same time, inhibitors of SCD, which prevent WNT labeling with exogenously provided palmitate (20), are inconsistent in their

activity against WNT signaling, suggesting that lipids other than palmitoleic acid may be incorporated (Fig. 2A). To directly probe the geometry of the acyl donor pocket in the PORCN active site, we labeled cells expressing a WNT3A fused with an Fc domain of IgG (WNT3A-Fc) with various exogenously supplied lipid probes that differ with respect to desaturation position and length, and that would enable subsequent copper-assisted cycloaddition of a biotin-conjugated azide to the alkyne group (Fig. 2, B and C). Although previous investigations into fatty acyl donor length preferences are consistent with those presented here showing PORCN favoring medium-length fatty acyl chains (20–23), we also observed PORCN preference for the position of desaturation (Fig. 2C). Indeed, moving the double bond in palmitoleic acid to the Δ7 and Δ11 positions in the case of C16:1n-9 and C16:1n-5, respectively, greatly diminished the ability to label WNT protein. In contrast, the HHAT acyltransferase did not exhibit any fatty acyl donor selectivity for acylating SHH-Fc protein in cultured cells. These data suggests that determinants within the PORCN site measure the distance of the double bond relative to the CoA group (Fig. 2D).

Bypassing PORCN fatty acyl donor stereoselectivity using an exogenously provided trans fat

A challenge to interrogating the role of the WNT lipid adduct in WNT manufacturing and signaling has been an inability to experimentally examine the consequences of immobilizing alternate fatty acids (FAs) onto WNT proteins. Our studies using PORCN from diverse animals species failed to delineate a strategy for attaching other fatty acids onto WNT proteins given the conservation of active-site features that enforce the use of a *cis* palmitoleic acid. A fatty acid with a *trans* double bond at the Δ9 position in a gauche-C11/12 conformation is topologically similar to *cis* palmiteolate and may serve as a weak PORCN fatty acyl donor (Fig. 3A). Although HHAT did not distinguish between the C16:1n-7 FA isomers, in agreement with its absence of a preference for a specific lipid, PORCN preferred the *cis* FA but nevertheless was able to make limited use of the *trans* molecule (Fig. 3A). An SCD inhibitor (A939572) blunted WNT acylation with a saturated fatty acyl probe (C16:0), consistent with previous observations (20), but not with the *cis* or *trans* unsaturated fatty acid probes, suggesting that the *trans* fat labeling was not enabled by cellular isomerization (Fig. 3A and Fig. S2) (24).

Using a pulse-labeling strategy with either *cis/trans* palmitoleic acid analogs, we observed a crippling effect of *trans*-Δ9 fatty acylation on WNT protein cellular release (Fig. 3, B–D). Given that the total amount of secreted WNT protein, however, was comparable between *cis* and *trans* fat–treated cells, we assume that the *trans* fat did not alter general protein secretion (Fig. 3C). This result also suggests that, indeed, a *trans* FA, and not a *cis* FA produced from an unknown *cis-trans* isomerase, was affixed onto the WNT protein. We also note that WNT974 inhibited *trans* fatty acylation of WNT protein, confirming the role of PORCN in this biochemical event (Fig. 3C). Although previous results have established the importance of lipidation in moving the WNT protein to the extracellular milieu, these observations are the first, to our knowledge, that demonstrate the essentiality of a *cis* double bond to WNT protein manufacturing.
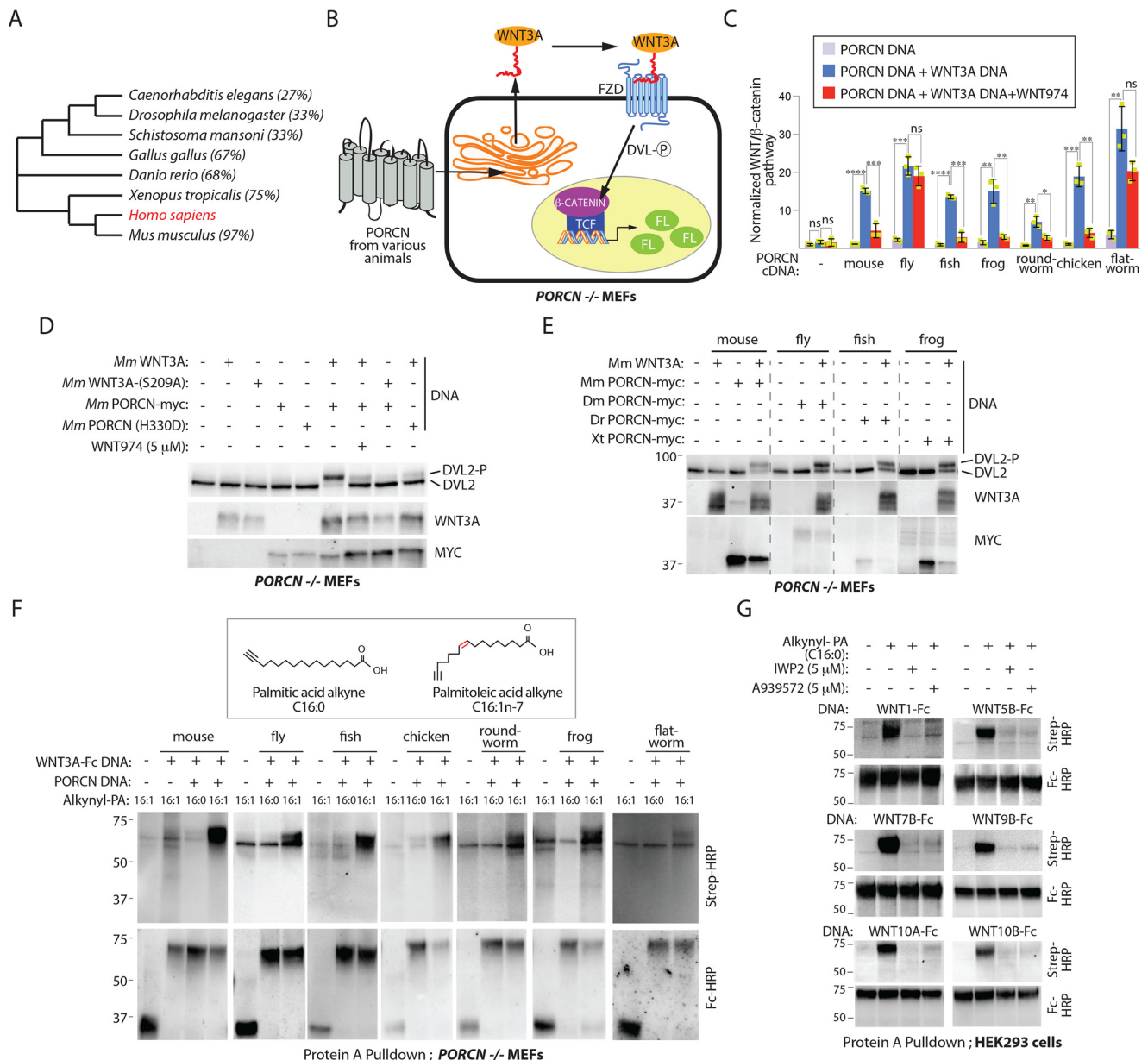
Figure 1. A universal requirement for *cis*-Δ9 fatty acids in metazoan WNT production. *A*, PORCN protein sequence divergence across metazoan phyla: Chordata (mouse (*Mus musculus*), zebrafish (*Danio rerio*), chicken (*G. gallus*), and frog (*Xenopus laevis*)), Nematoda (roundworm (*C. elegans*)), Platyhelminthes (flatworm (*S. mansoni*)), and Arthropoda (fruit fly (*Drosophila melanogaster*)). Percentages of amino acid sequence similarities of various PORCN enzymes relative to human PORCN are indicated in parentheses. The cladogram was generated using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/ (40)). (Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party–hosted site.) *B*, an assay for monitoring the activity of exogenously provided PORCN proteins. Activation of canonical WNT pathway results in nuclear accumulation of β-catenin, and induction of TCF/LEF transcription factor–associated target gene expression. A firefly luciferase (*FL*)-based reporter that measures β-catenin/TCF activity (SuperTopFlash) is used to monitor the activity of introduced PORCN DNAs in *PORCN*-null MEFs stably expressing murine WNT3A (PORCN$^{-/-}$, WNT3A, SuperTopFlash cells). *FZD*, Frizzled. *C*, PORCN from diverse animals reconstitutes mammalian WNT/β-catenin transcription. The PORCN inhibitor WNT974 (5 μM) exhibits WNT pathway–inhibitory activity. All *error bars* represent the mean of triplicates ± S.D. The experiment was repeated three times with similar results. *ns*, not significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. *D*, WNT3A-dependent DVL2 phosphorylation in MEFs devoid of PORCN expression was restored in cells transfected with a cDNA encoding a WT but not a catalytically inert (*H330D*) PORCN protein. WNT3A-(S209A) lacks the palmitoleation site. A PORCN inhibitor (WNT974, 5 μM) blocks WNT3A acylation. The experiment was repeated twice with similar results. *E*, introduced PORCN from various animals promotes WNT-mediated phosphorylation of DVL2 protein. The experiment was repeated twice with similar results. *F*, conservation of PORCN fatty acyl donor selectivity across metazoans. *PORCN*-null MEFs expressing WNT3A-Fc and the indicated PORCN proteins from various animals were treated with saturated alkynyl-palmitic acid (C16:0) or unsaturated alkynyl-palmitoleic acid alkynes (C16:1n-7). An IgG-Fc protein with a signal sequence served as a control (IgG-Fc). IgG-Fc or WNT3A-Fc protein immobilized on protein A–Sepharose was subjected to a copper-catalyzed alkynyl-azide cycloaddition reaction in the presence of biotin-azide, and lipidated proteins were detected using HRP-conjugated streptavidin. The experiment was repeated twice with similar results. *G*, a MUFA modification likely exists in all WNT proteins. A collection of WNT-Fc fusion proteins was used to determine the sensitivity of acylation (monitored using the click chemistry assay) in the presence or absence of a stearoyl-CoA desaturase inhibitor (A939572, 5 μM) or PORCN inhibitor (IWP2, 5 μM). The experiment was repeated twice with similar results.
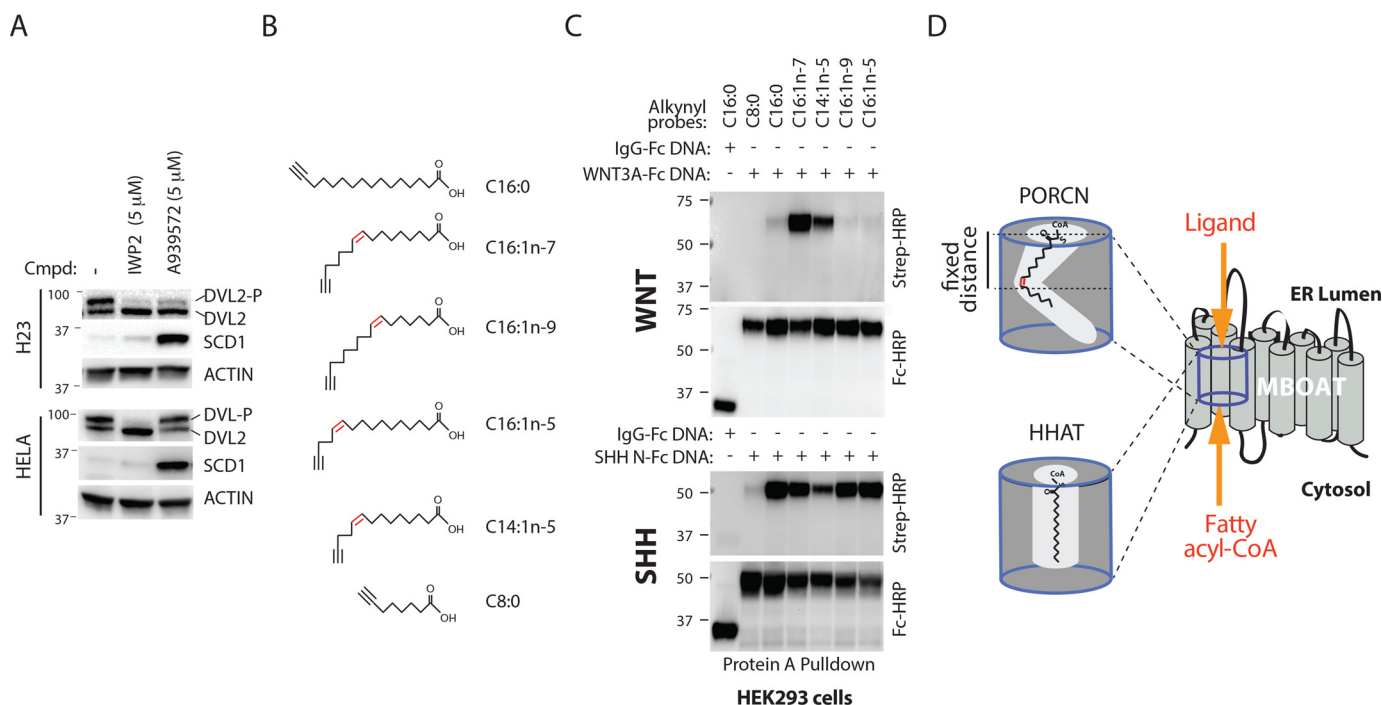
Figure 2. The active-site features of PORCN enforce *cis*-Δ9 palmitoleation on WNT proteins. *A*, an SCD inhibitor does not consistently block cell-autonomous WNT signaling in different cell lines. The lung cancer– derived H23 and cervical cancer– derived HeLa cell lines were treated with either IWP2 (5 μM) or A939572 (5 μM). Although in H23 cells both compounds (cmpds) were able to block WNT activity, as indicated by the reduction of phosphorylated DVL2 protein, A939572 was only active in H23 cells. The accumulation of SCD1 is presumably due to the pharmacoperone-like activity of the SCD inhibitors. The experiment was repeated twice with similar results. *B*, a collection of alkynylated FAs differing in carbon chain length and desaturation position. *C*, characterization of PORCN fatty acyl donor preferences using a click chemistry approach. Cells transfected with cDNA encoding either WNT3A or the N-terminal signaling domain of SHH fused with the IgG Fc domain (WNT3A-Fc and SHHN-Fc, respectively) were treated with various alkynyl FAs (illustrated in B) and subjected to a cycloaddition reaction as described previously. SHHN-Fc labeling served as a control for alkynyl probe cellular availability. The experiment was repeated twice with similar results. *D*, active-site models of HHAT and PORCN. The PORCN active site differs from HHAT in its ability to recognize the position of desaturation within the fatty acyl donor.

Trans fatty acylation compromises the ability of WNT proteins to extricate from PORCN

Substrate– enzyme interactions are often transient. However, we observed that WNT–PORCN complexes are detectable using biochemical approaches and, moreover, that this interaction is sensitive to PORCN and SCD inhibitors (Fig. 4, *A* and *B*). Thus, immobilization of a lipid on WNTs likely stabilizes WNT–PORCN complexes. To determine the molecular basis for failures in *trans* fatty–acylated WNT proteins to reach the extracellular milieu, we next measured the effects of exogenous *cis* and *trans* FA exposure on WNT–PORCN complexes. Cells fed *trans*-palmitoleate harbored more WNT–PORCN complexes than those exposed to *cis*-palmitoleate, suggesting that *trans* fatty–labeled WNTs are hindered in their capacity to unload from PORCN (Fig. 4, *C* and *D*).

Lipidation of WNTs is essential for their ability to interact with WLS and to exit the cell (25, 26). WLS expression is controlled by WNT/β-catenin signaling, supporting a transcription-based feedforward signaling mechanism (27). Here we show that forced expression of WNT proteins in HEK293 cells, including those that do not induce transcriptional responses in these cells, is matched by changes in the abundance of WLS protein, suggesting that the total levels of WNT production dictate the rate of WLS protein turnover (Fig. 4*E*). WLS in complexes with multiple WNT family members are eliminated in cells treated with PORCN inhibitors (Fig. 4, *F* and *G*). At the same time, these interactions were also sensitive to the presence of A939572, suggesting that the bulk of the palmitoleate used for WNT fatty acylation in this cell line is generated from cellular stores of palmitate (Fig. 4*F*). Given our observations that the act of lipidation results in WNTs becoming tethered to PORCN, it is conceivable that WLS requires this enrichment step to facilitate WNT–WLS interactions. Because of the entrapment of *trans* fatty–acylated WNTs on PORCN, it is technically not possible to study the effect of WNT *trans* fatty acylation on its binding ability to WLS. However, we reasoned that, similar to *trans*-labeled WNTs, we should also see increased WNT-PORCN complexes in cells devoid of WLS. We used CRISPR/Cas9 gene editing technology to generate *WLS* or *PORCN* KO subclones of the haploid cell line HAP1. Genomic DNA sequencing confirmed the presence of frameshift-inducing insertion/deletion mutations in the *PORCN* and *WLS* genes (Fig. 4, *H* and *I*). Elimination of PORCN or WLS failed to activate a WNT pathway reporter in the presence of WNT3A (Fig. 4*J*) and inhibited WNT cellular release (Fig. 4*K*). Inhibition of PORCN by WNT974 blocked both the WNT reporter and WNT secretion in WT HAP1 cells. Elimination of PORCN likely inhibits endogenous WNT fatty acylation, resulting in decreased WLS levels in *PORCN* KO HAP1 cells (Fig. 4, *K* and *E*). The absence of WLS also halted the transfer of acylated WNTs from PORCN and resulted in increased WNT–PORCN complex accumulation (Fig. 4*L*).
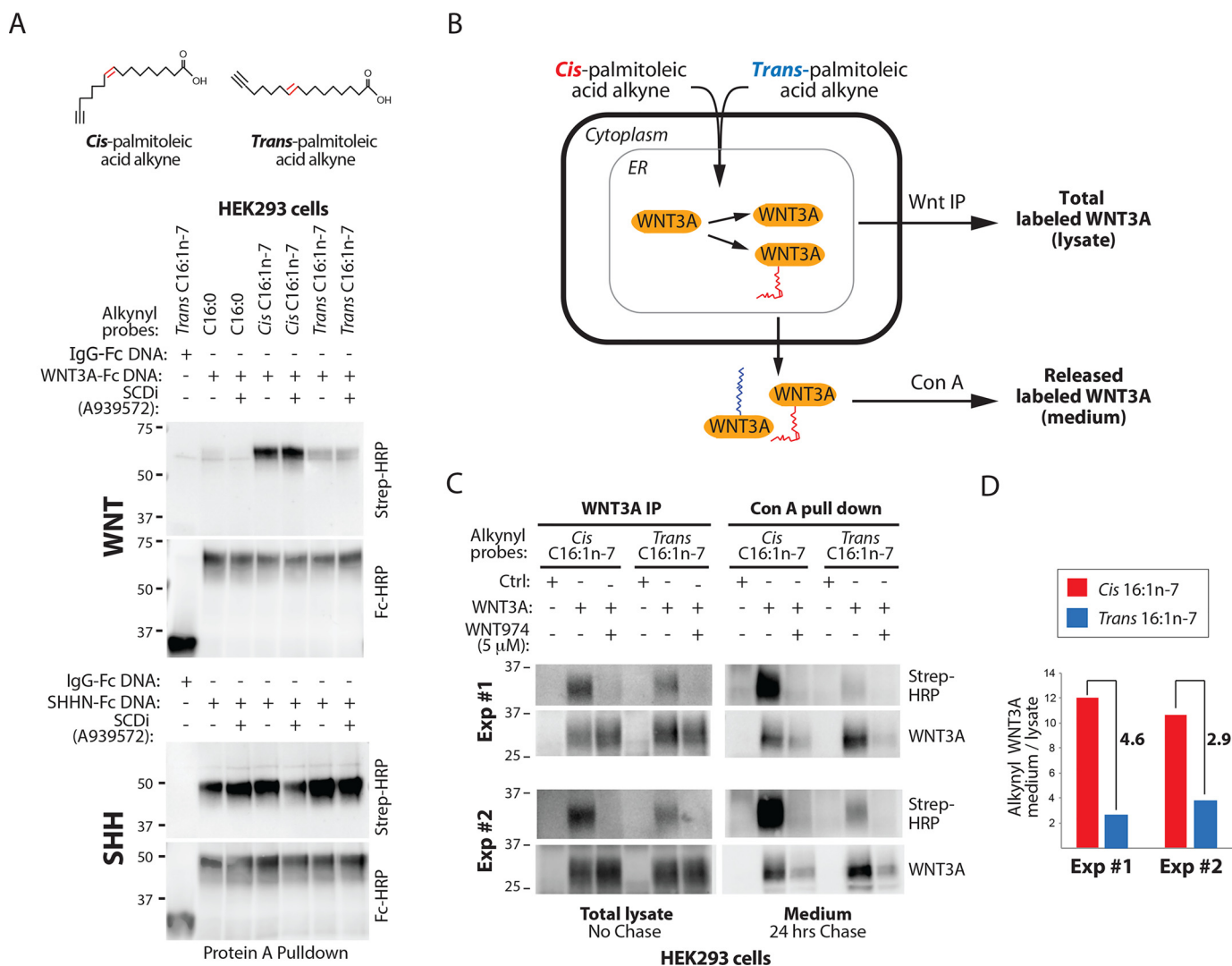
Figure 3. WNT molecules labeled with *trans* palmitoleic acid fail to leave the secretory pathway. *A*, PORCN exhibits stereoselectivity for its fatty acyl donor. *Cis* and *trans* alkynlated palmitoleic acids (C16:1n-7) were used to label WNT-Fc or SHHN-Fc proteins. A stearoyl-CoA desaturase inhibitor (*SCDi*, A939572, 5 μM) was used to prevent cellular isomerization of alkynylated probes. SHHN-Fc labeling served as a control for alkynyl probe cellular availability. The experiment was repeated three times with similar results. *B*, a click chemistry–based strategy for investigating the influence of palmitoleic acid isomerization on WNT cellular release. *IP*, immunoprecipitation. *C*, cell medium was collected 24 h following pulse-labeling of HEK293 cells with either *cis* or *trans* alkynylated palmitoleic acid. WNT proteins from the culture medium were enriched using ConA-Sepharose beads and subjected to a cycloaddition reaction. A baseline labeling efficiency associated with each palmitoleate isomer was determined using a similar analysis of WNT protein isolated from total lysate. The experiment (*Exp*) was repeated twice with similar results. *Ctrl*, control. *D*, the values for total and released click chemistry–labeled WNT proteins were used to calculate the normalized WNT secretion value for each palmitoleate isomer. In two separate experiments, WNT protein release is compromised when it is adducted to a *trans* palmitoleate.

Discussion

The dearth of salient features in aliphatic chains that could be exploited by proteins for selective engagement of lipids poses a physical challenge to the inclusion of FAs as intermediaries in signal transduction. Here we demonstrate that a *cis*-Δ9 MUFA is the critical FA for WNT release from PORCN, extending the previously established importance of this lipid in the WNT protein lifecycle (summarized in Fig. 5). The *cis* palmitoleate with its kink in the Δ9 position may lower the energy requirements for either their diffusion away from PORCN before binding to WLS or WLS-mediated extrication from the PORCN active site. The interaction of WLS and PORCN suggests that the latter mechanism is more likely to be the case (28).

Post-WNT production, the kink in palmitoleate is likely responsible for the ability of WNT proteins to induce dimeriza-

tion of the extracellular domains of Frizzled receptors (29). At the same time, the WNT deacylase *NOTUM* incorporates several residues within its active site as part of a steric gate that precludes the binding of FAs without a *cis*-Δ9 double bond (30). Our finding that conserved features in the active site of PORCN enforce the addition of a *cis*-Δ9 MUFA onto WNT proteins in likely all animals reinforces the importance of this molecular kink in WNT signaling. The delineation of a lipid bilayer-spanning tunnel created by a ring of helices revealed in the first crystal structure of an MBOAT family member (DltB from Gram-positive bacteria) could help to identify potential residues that contribute to PORCN fatty acyl donor selectivity (31).

The influence of fatty acyl protein modifications on WNT signaling is pervasive and extends beyond the biosynthetic steps
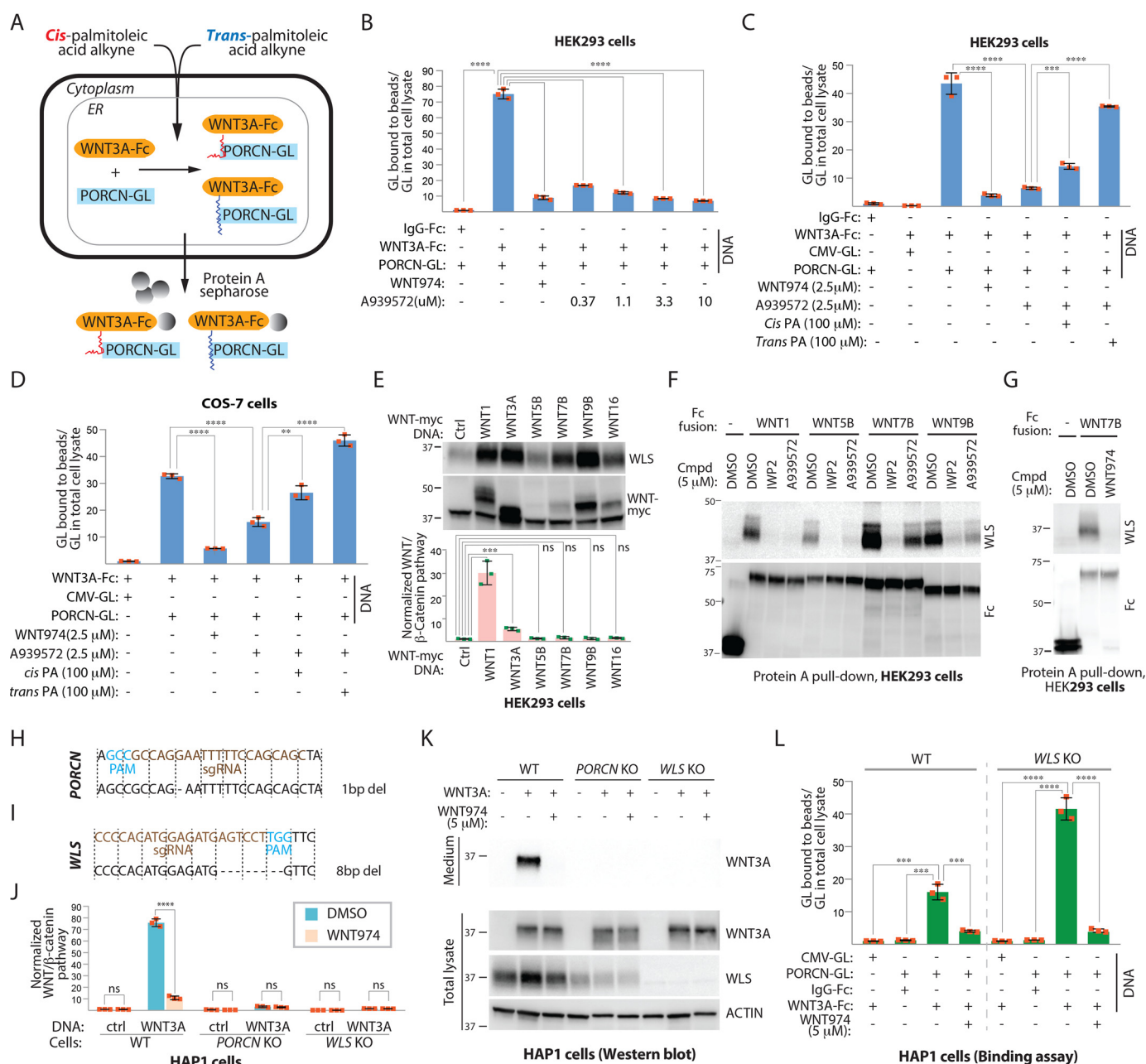
Figure 4. *Trans* fatty acylation hinders WNT extrication from PORCN. *A*, a quantitative assay for measuring PORCN and WNT interactions in cells treated with *cis* or *trans* palmitoleate. HEK293 cells transiently transfected with PORCN-GL and WNT3A-Fc DNA were treated with *cis* or *trans* palmitoleate for 24 h and then lysed. PORCN-GL activity associated with WNT3A-Fc bound to protein A-Sepharose beads was then determined. The PORCN-GL signal from the total cell lysate was used for normalizing the PORCN-GL signal to WNT3A-Fc. *ER*, endoplasmic reticulum. *B*, A939572 inhibits the interaction between PORCN-GL and WNT3A-Fc in a dose-dependent manner. ****, $p < 0.0001$. *C*, accumulation of WNT-PORCN intermediates with WNT *trans* fatty acylation. Shown are results of the assay described in *A* with cells treated with or without A939572 (2.5 μM) or WNT974 (2.5 μM). ***, $p < 0.001$; ****, $p < 0.0001$. *D*, modification of WNTs with a *trans* fat increases WNT–PORCN complex formation in COS7 cells. **, $p < 0.01$; ****, $p < 0.0001$. All *error bars* in *B–D* represent the mean of triplicates ± S.D. Each experiment was repeated twice with similar results. *E*, WLS protein abundance is gated by WNT ligands in a β-catenin/TCF–independent fashion. Shown is expression of WNT-myc proteins that induce a β-catenin/TCF transcriptional response (WNT1, WNT3A) and that do not induce a β-catenin/TCF transcriptional response (WNT5B, WNT7B, WNT9B WNT16) stabilize the WLS protein. The experiment was repeated twice with similar results. *Ctrl*, control. *ns*, not significant. ***, $p < 0.001$. *F*, the WLS chaperone protein is a sensor for WNT protein abundance. Several WNT-Fc proteins bind to endogenous WLS protein in a PORCN inhibitor– and SCD inhibitor–sensitive manner. The experiment was repeated twice with similar results. *G*, the PORCN inhibitor WNT974 disrupts the WNT–WLS interaction. *H*, generation of *PORCN* KO HAP1 cells using the CRISPR/Cas9 system. *PORCN* null HAP1 cells contain 1-bp deletion (*del*) in the *PORCN* gene. *I*, generation of *WLS* KO HAP1 cells using the CRISPR/Cas9 system. *WLS* null HAP1 cells contain 8-bp deletion (*del*) in the *WLS* gene. *J*, *PORCN* and *WLS* KO HAP1 cells have compromised WNT/β-catenin pathway activity. WNT974 blocks WNT3A-mediated transcriptional activation in WT HAP1 cells. The experiment was repeated three times with similar results. ****, $p < 0.0001$. *K*, WNT proteins fail to leave the secretory pathway in the absence of PORCN or WLS. The experiment was repeated twice with similar results. *L*, an increase in WNT-PORCN complex abundance in HAP1 cells lacking WLS. The PORCN-GL signal from the total cell lysate was used for normalizing the PORCN-GL signal bound to WNT3A-Fc. The experiment was repeated three times with similar results. ***, $p < 0.001$; ****, $p < 0.0001$.

involved in the manufacturing of WNTs. For example, the ability of LRP6 to exit the ER is gated by palmitoylation of a juxtamembrane residue (32), and the activity of PORCN itself is controlled by palmitoylation by an unknown intracellular enzyme (22). The extensive interconnectivity of lipid metabolism and WNT signaling may have its roots in coopting a lipid
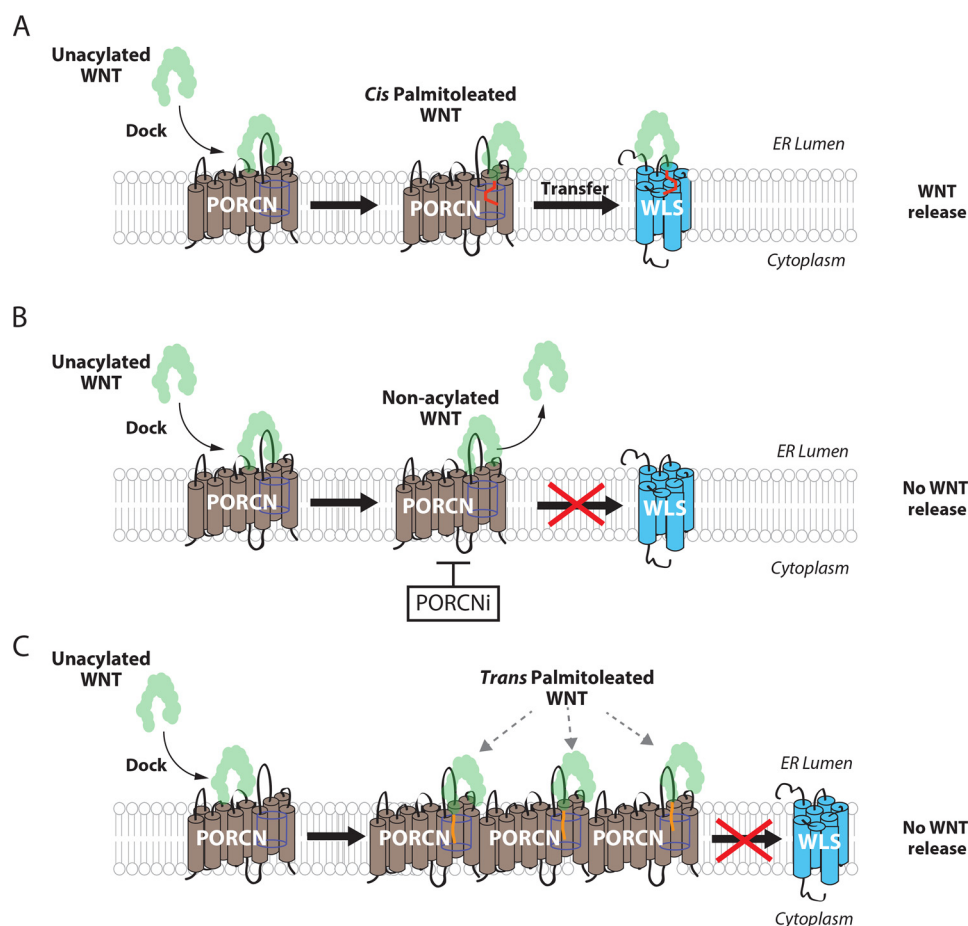
Figure 5. Fatty acyl species-selectivity checkpoints in WNT production. *A*, PORCN active-site features conserved across animals favor *cis*-Δ9 fatty acyl CoA substrates, an adduct on WNTs that is essential for their extrication from PORCN, presumably by WLS. *ER*, endoplasmic reticulum. *B*, active-site features that govern PORCN-mediated fatty acyl selectivity are likely attacked by PORCN inhibitors. *C*, addition of a *trans* fat to unacylated WNTs result in accumulation of PORCN–WNT complexes, presumably because of their inability to transfer to WLS.

sensing apparatus in unicellular organisms for the purpose of intercellular communication (33). At least in humans, palmitoleate is the second most abundant MUFA in adipose tissue and blood (34) and perhaps serves in the PORCN–WLS system as currency for synchronizing metabolic health and tissue renewal. Indeed, loss of SCD in induced pluripotent stem cells elicits a transcriptional response that includes the induction of many WNT genes, suggesting hardwiring of MUFA abundance with WNT signaling (35).

Based on our observations with a *trans* fatty–acylated WNT molecule, we assume that WNT proteins modified with a saturated FA similarly would not be readily extricated by WLS and, thus, destined for entrapment in the secretory pathway. Given that exogenously supplied lipids can influence octanoylation of the appetite-controlling hormone ghrelin by its acyltransferase ghrelin *O*-acyltranferase (36), we suspect that dietary contributions of saturated and *trans* fats might influence the efficiency of WNT production in adult cells.

Despite their chemical diversity, the majority of PORCN inhibitors likely target the PORCN active site based on their ability to successfully compete with a fluorescently labeled active-site probe for PORCN binding (18). For antagonists such as GNF-1331 (the precursor of WNT974) and IWP2, which have also been evaluated for their activity against other MBOATs, there is evidence that these molecules exhibit selectivity for PORCN (18, 37). Our observations described here provide insights into how this is achieved; determinants that support PORCN selectivity for *cis*-Δ9 fatty acyl donors can be exploited to achieve chemical control of PORCN. Additional support for this conclusion may be found in enantiomer-dependent activity of at least one class of PORCN inhibitors (38). The PORCN–WLS relationship likely extends throughout the animal kingdom, based on our evidence of the conservation of determinants that enforce *cis*-Δ9 acylation of WNT proteins and that mammalian WNT proteins expressed in *Drosophila* are not released in the absence of WLS (25). Thus, PORCN inhibitors disrupt an ancient partnership between PORCN and WLS that likely was forged to overcome biophysical and protein design constraints associated with the use of lipid intermediaries for signal transduction. At the same time, the inability of WNT974 to disable a subset of PORCN from the animal sources tested, such as *Drosophila* and *Schistosoma* (Fig. 1C), further suggests that some nonconserved features found in the PORCN active site could be exploited for species-specific disruption of animal development for therapeutic development against parasites that afflict a variety of host animals including humans, livestock, and pets.

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Palmitoleation is essential for WNT extrication from PORCN

Experimental procedures

Cell culture and reagents

$PORCN^{-/-}$ MEFs were provided by Charles Murtaugh (University of Utah). HEK293, COS7, and HeLa cells were purchased from the ATCC. HAP1 cells were purchased from Horizon Discovery. The *Gallus gallus* PORCN construct was provided by Laura Burrus. The *Caenorhabditis elegans* PORCN construct was provided by Rueyling Lin. The *Xenopus tropicalis* PORCN construct was purchased from Transomic Technologies. Neobiolab synthesized a humanized *Schistosoma mansoni* PORCN-myc construct with the following sequence: GCGGCCGCATGGACGAGCAGGAGGAGTCTTGGTACATGGACGAGGACGAGGAGGAGCTGTTCGAGGAAGTGGACGAGGAGAACGGCGATAGCGATCTGAGCGGCAACTACCTGGACGACCTGGAAACCGTGGAGGAAGACGACGGCGATTTCGAGGACGTGTCCTTCGAGAACGCCCTGAGCATGAAGAGCATCAGCAGCGAGATCCTGTCTTGCAGCATCCCCGTGCTGAAGCAGGCCTATTGGCTGCTGATCCTGATCCTGGTGTCTTGCCTCATCTGGAGGGTGGTGCAGTTCCTGCTGAATCGCTGCTTCCCCAGCAACGGCAAGATCTCTCGGACCTTCGTGGCCATCATCCTGCACATCTTCAGCATTTGCCTGGGCCTGCTGGTGCTGTACTACGCCTCCTACGACCTCTGGTGGATCGTGATCGGCCTGATCATCACCCTGACCCTGCTGTTCACCATCAACATCAACAGCGACATCAGCAACAGCCACGGCGAGTACACCAATTGGGAGCTGTTCGTGACCATCGGCATTTGCAGCAGCGTGCAGCTGTATTGCGAGTTCTACAAGAACCCAGTCAAGTGGCACCAGATCAGAGGCAGCATCATGATCATCATCATGAAGTGCATCAGCTTCAGCATGGAGAACAAGAACTTCTACAAGACCGTGAACAAGGAGCCTTGTTGCATCCTCCTCTGGACCCCTCTGTATAGAGGCCTGCTGTGGCTGAGCTATTGCCTCTGTCCAGCCAGCCTGCTGTTCGGACCTTGGTTCAACCCCCTGCGCTACGAGGAGATGGTCCGGAACTACGCAGTGAACGACCGGCCCTTCAGCTTCAAGAACACCATGATCAGCCTGTTCCAGACCGCCAAGCTGTTCGGCCTGAGCTTCCTGTACCTGACCTACAGCACTTGCTTCACCGACACCCTGGCCAGCATCCTGAAACTGCAGCCTTGGCTGCACGCCTACTTTGCCAGCCAGAGCTTCCGGTTCAGCCACTACTTCATCTGCATCAGCAGCGAGAGCTCTATGACCGCCCTGGGCTATTGCGACAAGTACAGCATCCAGAGCAACAAGCAGGAGAACACCAAGGAGAAGAACCGGAGCAAGGACGTGTACAAGCCAGTGGTGGTGACCAGGCCTCTGTTCATCGAGTTCCCCAGGAGCCTGGTCGAGGTCGTGATCTATTGGAACCTGCCCATGCACACTTGGCTGAAGCAGTACGTGTACAAGCCTCTGAGACCTTACGGCCACGTGTACGCAGTGCTGGGAACATACACAGCCAGCAGCCTGCTGCACGGACTGAACTTCCAGCTGAGCGCAGTGCTGTTCTCCATCGGCATCTACGCCTACACCGAGTACGTGTTCCGGGAGAAGCTGGCTAAGATCCTGGACGCTTGCATCGCCAGCAGGCCTTGCCCCGAGAGTTGTTGCCACTCCAACAAGAACAGCAGCTGGGTCTACTGGTGCAATGTGGCCTTCTCTTGCCTGGCCATCTTCCACCTGGCCTACCTGGCCGTGATGTTCGATACCAGCGAGCAGCAGTTCCAGGGCTACAACATGTGGCACACCATGAACAAGTGGTACAACCTGGGCTTCCTGTCTCACATCGTGGCCTTTGCCAACTTCCTGTTCTACCTGTACCTGGCGGCCG-

CTCGAGGTCACCCATTCGAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCATACCGGTCATCATCACCATCACCATTGA.

Chemicals

IWP2 was synthesized as described previously ([17]). WNT974 was purchased from Active BioChem, and the SCD inhibitor A939572 was purchased from Biofine International. Biotin-azide and click chemistry reaction buffers were purchased from Invitrogen. *Cis* and *trans* ω-alkynyl fatty acid probes were synthesized by Dr. Chuo Chen's group and are described in the supporting experimental procedures.

Western blot analysis

Cell lysates were generated with PBS/1% NP40 buffer supplemented with protease inhibitor mixture (Sigma, catalog no. S8820). Protein sample loading buffer was added to cell lysates, and proteins were separated on SDS-PAGE (Bio-Rad Criterion TGX Precast Gel). Antibodies used for immunoblotting were purchased from the following sources: DVL2 (catalog no. 3216), WNT3A (catalog no. 2721), MYC tag (catalog no. 2272), and SCD1 (catalog no. 2794) from Cell Signaling Technology; WLS/GPR177 (catalog no. MABS87) from Millipore; actin (catalog no. A2228 from Sigma); Fc-HRP (catalog no. sc-2453) from Santa Cruz Biotechnology; and streptavidin–HRP (catalog no. 405210) from Biolegend.

Luciferase reporter assay

The reporter assay was executed as described using a Dual-Luciferase kit (Promega). SuperTopFlash and control SV40-driven *Renilla* luciferase reporters used in reporter assays were described previously ([39]).

Click chemistry assay

HEK293 cells were transiently transfected with either the IgG-Fc, WNT3A-Fc, or the SHH N-Fc DNA expression construct and treated with various ω-alkynyl fatty acid probes (100 μM final concentration) for 6 h. WNT-Fc and SHH-Fc proteins modified with the synthetic alkynylated fatty acid probes were then purified from the lysate using Protein A–Sepharose and subjected to a copper-catalyzed alkyne-azide cycloaddition reaction in the presence of biotin-azide. The biotinylated WNT3A-Fc and SHHN-Fc proteins were run on an SDS-PAGE gel and detected using streptavidin–HRP. The expression of total IgG-Fc, WNT3A-Fc, and SHHN-Fc proteins was evaluated using anti-Fc IgG cross-linked to HRP.

WNT secretion assay

HAP1 cells (WT, *PORCN* KO, and *WLS* KO) transiently transfected with either the control or the WNT3A cDNAs were treated with DMSO or WNT974 (5 μM) for 48 h. Total secreted WNT3A proteins from the culture medium were enriched on ConA–Sepharose. WNT3A immobilized on the Sepharose beads was extracted by adding sample loading buffer and heating the beads at 95 °C for 2 min. Protein was separated on SDS-PAGE and detected using a WNT3A antibody.

Pulse–chase assay to monitor secretion of acylated WNT proteins

HEK293 cells transiently transfected with WNT3A cDNA were labeled with either *cis* or *trans* alkynylated palmitoleic acid for 6 h. The cells were thoroughly washed with PBS to remove any remaining fatty acid isomers after the end of the labeling period and replenished with fresh culture medium. The WNT proteins labeled with alkynylated probes were collected from the medium after 24 h (immobilized on ConA-Sepharose) or total lysate (immobilized on protein A–Sepharose using a WNT3A antibody). A click chemistry reaction was performed on the Sepharose-bound WNT3A proteins, and the palmitoleated WNT proteins were detected using streptavidin-HRP. The total WNT3A proteins isolated from either of the sources were detected using a WNT3A antibody. The value for total acylated WNTs in lysate was used for normalizing the WNT secretion value derived from the medium.

Binding assay for measuring WNT-PORCN interaction

HEK293 cells were transiently transfected with WNT3A-Fc and PORCN-GL cDNA constructs. 24 h post-transfection, the cell culture medium was changed to low-serum medium (1% FBS) along with either *cis* or *trans* alkynylated palmitoleic acid. To avoid isomerization of the fatty acyl probes, cells were also treated with 2.5 μM A939572 (an SCD inhibitor) along with the exogenously supplied FA probes. After 24-h treatment, cell lysates were generated and incubated with protein A–Sepharose for 6 h in a vertical rotator. After several washes, *Gaussia* luciferase (GL) signals associated with the Sepharose beads were determined. The GL signal from the total cell lysate was used for normalizing the luciferase signal bound to WNT3A-Fc.

Statistics and reproducibility

The number of times an experiment was repeated is indicated in the figure legends. All experiments were repeated at least twice. Quantitative data are presented as mean $\pm$ S.D. from three independent measurements. Statistical testing was performed using Student's *t* test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

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